WATER HOMEOSTASIS: EVOLUTIONARY MEDICINE

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ABSTRACT

As a major component of homeostasis, all organisms regulate the water composition of various compartments. Through the selective use of barrier membranes and surface glycoproteins, as well as aquaporin water channels, organisms ranging from *Archaebacteria* to humans can vary water permeabilities across their cell membranes by 4 to 5 orders of magnitude. In barrier epithelia the outer, or exofacial, leaflet acts as the main resistor to water flow; this leaflet restricts water flow by minimizing the surface area of lipid molecules which is not covered by phosphate headgroups and by packing hydrocarbon chains at maximal density. Cells may enhance the barrier by expressing glycoproteins that augment the "thickness" of unstirred layers at their surfaces, reducing osmotic gradients at the lipid bilayer surface. Aquaporins markedly and highly selectively accelerate water flux and are "switched on" either by deployment into membranes or gating. This review summarizes these mechanisms in many species, and indicates potential roles for manipulating water permeabilities in treating disease.

As first articulated by Claude Bernard (1), the ability of organisms to survive free of the oceans we evolved from requires the rigorous maintenance of stable chemistries within the "milieu interieur" that bathes our cells. The most fundamental form of this constancy of composition, or homeostasis, is the maintenance of osmolality. To control tightly the osmolalities of various compartments, organisms regulate the rates of water flux across epithelial layers, varying permeability coefficients over 4 orders of magnitude, from levels of 10^{-5} cm/sec to values higher than 10^{-1} cm/sec (2). Although water is freely permeable across most cells, organisms minimize water fluxes by creating barrier epithelia that combine elements of lipid structure, dense arrays of membrane proteins, and the maintenance of unstirred layers (regions of the liquid phase adjacent to the membrane that have a composition different from the bulk solution).

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Organisms speed water flow by expressing aquaporin water channels in specific membranes. This review will summarize our current understanding of how barrier epithelia limit, and aquaporins accelerate, water flux.

BARRIER EPITHELIA

Although water is generally freely permeable across cell layers, some specialized epithelia limit water flux to permeability values in the 10^{-5} to 10^{-4} cm/sec range (2). Measured permeability coefficients and the roles of selected epithelia in maintaining osmotic homeostasis are listed in Table 1. For nearly all epithelia, the apical membranes serve as the main barrier to water flux. Our studies have shown that epithelia use several strategies to reduce water flux across the apical barrier. These include specialized lipid structures that feature asymmetric distribution of lipid constituents in the exofacial, as opposed to the cytoplasmic, leaflet, which structure the exofacial leaflet to reduce surface area available for diffusion and tight packing of hydrocarbon chains to reduce diffusion within the bilayer. Membrane proteins, which can comprise over half of the surface area of membranes, may also augment barrier function. Finally, cells can express abundant mucins and other proteins that create a large unstirred layer on the apical surface.

Bilayer Asymmetry: Barriers in Series

Studies of membrane bilayer structure have shown striking asymmetries in the distribution of lipid substituents between the exofacial and cytoplasmic leaflets of the bilayer (3, 4). The exofacial leaflets

TABLE 1
Permeabilities of Barrier Epithelia and Apical Membrane Vesicles Isolated From Them

Epithelium*	P_{H2O} (cm/sec $\times 10^4$)
Intact mammalian bladder (15, 53)	0.41
Bladder apical membranes (54)	2.3
Thick ascending limb (55)	15
Thick ascending limb apical membranes (56)	33.5
Toad bladder (no ADH) (17)	0.8–2.5
Toad bladder apical membranes (57)	3.9
Teleost gill (58)	0.15
Teleost gill apical membranes (58)	7.4
Gastric endosomes (59)	2.8
MDCK cells (8)	1.8–3.5
Cortical collecting duct (no ADH) (2) 20	

*References for permeability values are provided in parentheses.

contain predominantly phosphatidylcholine, whereas the inner leaflets contain phosphatidylserine and phosphatidylethanolamine. Outer leaflets contain abundant sphingomyelin, which, by binding tightly to cholesterol, traps high levels of cholesterol in the outer leaflet, leaving little in the inner leaflet (3, 4). Developing and maintaining this asymmetric distribution of lipids requires that cells produce membrane in an asymmetric manner in the Golgi, and that they expend metabolic energy by using phospholipid flippases to move molecules like sphingomyelin from inner to outer leaflets (5). Is there a relationship between these structural differences and the barrier function of each leaflet? Testing this required that we create asymmetric bilayers in the laboratory. First, we measured water permeabilities in liposomes in which one leaflet was rigidified using Praesodinium, at varying temperatures (6). These studies showed that rigidification of a single leaflet led to marked decrease in water permeability, and indicated that each leaflet is an independent resistor to permeation. To test this, we prepared lipid bilayers in Ussing chambers into which we added cholesterol sulfate (which, unlike cholesterol, cannot cross the bilayer) to one leaflet, both leaflets, or to no leaflets, and measured the water permeabilities (7). Adding cholesterol sulfate to one leaflet reduced permeability; adding it to both reduced permeability further. Modeling the bilayer leaflets as resistors in series predicted accurately the permeability measured when both leaflets contained cholesterol sulfate. To test this notion in living cells, we prepared liposomes which matched closely the compositions, respectively, of the exofacial and cytoplasmic leaflets of MDCK Type 2 cells, and measured their water permeabilities (8). From these values we modeled the expected permeability of the MDCK cell layer, and found that our predicted water permeability matched closely the permeability we measured when we placed the cells into an Ussing chamber. These studies showed that bilayer leaflets act as resistors to permeation in series. When cells cluster phosphatidyl choline, sphingomyelin, and cholesterol in the outer leaflet, they create an effective barrier to water flux.

How Does the Apical Leaflet Function as a Barrier?

We have performed numerous studies in which we measured water permeabilities of varying lipid structures. These studies showed that reducing overall membrane fluidity reduced permeation, indicating a role for the mobility of hydrocarbon chains in determining water permeability (9–11). Recently, we have teamed up with John Nagle and

Stephanie Tristram-Nagle, physicists at Carnegie-Mellon, to perform detailed neutron and X-ray diffraction structural studies on lipid bilayers, and to compare the structures to permeabilities (12, 13). We found that bending modulus and other physical features of bilayers did not correlate with permeability. By contrast, packing density did. We have developed a novel theory of how these leaflets function as barriers, shown in Figure 1. In this formulation, the phospholipid headgroup is an absolute barrier to permeation. When the phospholipid molecules are densely packed, there is little space between headgroups, and the ability of water to diffuse into the core of the lipid bilayer is limited. In this respect, it is interesting that cells concentrate phosphatidylcholine in the outer leaflet. Space-occupying models of phosphatidylcholine show that the choline headgroup, which predom-

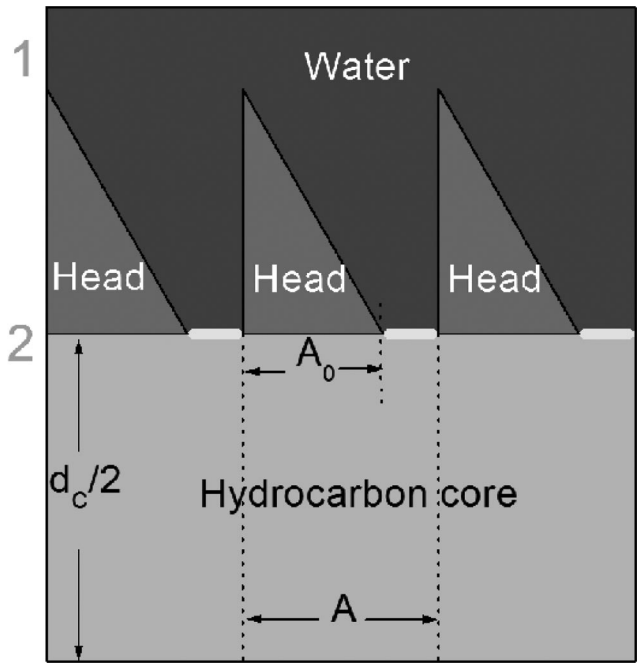

FIG. 1. Model of outer leaflet of barrier apical membrane showing features that restrict water movement. Phospholipid headgroups (*red*) completely block entry of water into the hydrocarbon core. The density of packing of individual phospholipids, combined with the bulkiness of the headgroups, can leave little free space (*yellow*) for water to reach the hydrocarbon core. The total area of each phospholipid molecule, A, minus the area of the headgroup, A_0, equals the yellow area that is available for water entry. When cholesterol is present, the hydrocarbon core of the outer leaflet, of thickness $d_c/2$ (diameter of the core of the bilayer divided by 2) presents an additional barrier to water flux. See text and Nagle et al (12) and Mathai et al (13) for details.

inates markedly in the exofacial leaflet, occupies more space than the serine or ethanolamine headgroups, which are most common in the cytoplasmic leaflet. On this basis, dense packing of the relatively bulky choline headgroups restricts access of water to the core lipid of the outer leaflet.

Structural studies have also shown a role for cholesterol. At any given packing density, addition of cholesterol to the bilayer markedly reduces permeability (12, 13). This results from the ability of cholesterol to bind, through tight hydrophobic interactions, to the hydrocarbon chains in the core of the outer leaflet, restricting their movement. Because water diffusion through the hydrocarbon core requires movement of the chains away from the polarized hydrogen-oxygen bond, reduced mobility reduces markedly the diffusion rate of water through the hydrocarbon. In this manner, when cells load the outer leaflet with sphingomyelin, they force a pronounced concentration of cholesterol in this leaflet. The cholesterol restricts movement of the hydrocarbon chains and reduces markedly the rate of diffusion of water through the hydrocarbon. It is notable as well that these same mechanisms, increased packing and cholesterol restriction of diffusion, also define how outer leaflets restrict the fluxes of small solutes such as urea, glycerol, and ammonia (14). Interestingly, proton flux is reduced by tight packing of lipids, but cholesterol-induced restriction of hydrocarbon chain movement is associated with increased rather than reduced proton flux.

A role for integral membrane proteins in barrier function? To determine if membrane proteins can serve as essential components of the barrier, we measured water permeabilities of intact mouse bladder epithelium (urothelium) (15). This transitional epithelium features large arrays of integral proteins called uroplakins in its apical membrane, with more than 85% of the surface occupied by paracrystalline arrays of these proteins. Because urothelium exhibits remarkably low water permeabilities (Table 1), we reasoned that uroplakins might play an important role in barrier function. We obtained uroplakin knockout mice from T.T. Sun and found that water permeability was indeed 10 times higher in the knockout, as compared to the wild-type animals (15). Urea permeability was also increased in the knockouts. These results demonstrated an essential role for uroplakins in barrier function. We still do not know whether the uroplakins alter the lipid structure or act in a barrier capacity.

Do barrier epithelia create deep unstirred layers to augment barrier function? Table 1 compares water permeabilities of intact epithelia studied in Ussing chambers with the permeabilities of

apical membrane vesicles from the same epithelia. It is notable that water permeabilities of gill and mammalian bladder are 5- to 30-fold less than the permeabilities of the corresponding vesicles. These results suggest that the intact epithelium has constructed unstirred layers to reduce water permeation. How do unstirred layers work? The flux equation:

Flux (mole/sec) = Permeability (cm/sec) × Surface Area (cm^2) × Concentration Gradient (mole/cm^3)

shows that flux can be decreased by reducing the permeability coefficient, by reducing the surface area available, or by reducing the concentration gradient. Unstirred layers reduce markedly the concentration gradient at the membrane by slowing the rate of diffusion of water from the bulk solution to the solution immediately adjacent to the membrane. This reduced gradient slows the flux rate. Such a mechanism is well-known to operate in the gastric mucosa, within which a thick mucus layer creates a pH gradient between the bulk gastric juice, at a pH of 2; and the surface of epithelial cells, in which the pH is close to 7 (16). Epithelia might create unstirred layers by trapping the water layers close to the membrane surface using mucins and other highly branched, and charged molecules. Some evidence suggests that the urothelia and gill may create elaborate glycosaminoglycan layers that reduce osmotic gradients. We have begun to study the role of these mucins in epithelial barrier function using mice in which urothelial GAG production is ablated genetically, cultured epithelial cells are either expressing or not expressing abundant mucins, and using direct measurements of the barrier properties of different forms of mucins, under varying conditions.

AQUAPORINS

The Search for the Water Channel

Studies in intact organisms and intact cells had established that several cell types exhibited remarkably high water flux across their membranes. Prominent cells in early studies included the red blood cell, proximal tubule and collecting duct from the mammalian kidney, and toad urinary bladder (reviewed in 17). Ingenious biophysical measurements revealed that the water flux in these cells exhibited high water permeabilities, in the range of 0.1 to 0.01 cm/sec, and that the permeabilities were often selective for water over other substances (17). Water flux in these cells was found to be inhibited by mercurials, and that the inhibition was reversible with sulfhydryl reagents, indi-

cating that a protein was involved (17). When it was shown that the activation energy of water diffusion in aqueous solutions had a low activation energy (in the vicinity of 2 to 4 kCal/mole/°K), measurements of water flux into and across these cell types were performed at varying temperatures, and the activation energy for water flow across these putative water channels was found to be equally low (17). This suggested that, similar to the bacterial water and ion channel, gramicidin, the channel was of high conductance and was also highly selective for water over other substances (17). The low activation energy suggested that water crossing the pore was in contact with other water molecules, or with amino acids which resemble water in their binding to water in the pore.

Efforts to identify the water channel continued, and included some from our own laboratory, in which we showed that the glucose carrier Glut 1 could conduct water, but was not the water channel (18). In 1992, Peter Agre identified a novel 28-kb protein that appeared as an impurity in his preparations of other red cell membrane proteins (19). The protein was very abundant, and was expressed at levels close to those anticipated for the red cell water channel. Using sequence data he cloned the protein (20). At the suggestion of his prior mentor, John Parker, who thought that this protein might be the water channel, he expressed the protein in *Xenopus* oocytes and found that it markedly increased their water permeability (21).

Successful Reconstitution and Definition of Functional and Structural Properties

Peter then sent the protein to me, and I reconstituted it into proteoliposomes and measured the water permeabilities of liposomes lacking the protein and proteoliposomes containing it (22). The protein markedly increased the water permeability of the liposomes (22–24). From the amount of protein expressed and the permeability, we obtained a conductance for water of 5.43×10^{-14} cm^3/sec, if we assumed that the functional pores were the 4 monomers in each tetramer (22–24). If the functional unit was the tetramer, then the conductance value would be four times higher. Because the monomer value resembled closely the water conductance for the bacterial pore, gramicidin, we concluded that the functional unit is a monomer (22). Given the known amount of aquaporin expression on the erythrocyte membrane, this conductance showed that water permeability across red cell membranes could be accounted for entirely by aquaporin 1, as the protein came to be called. This result was confirmed in later studies of a woman with a homozy-

gous knockout of aquaporin 1; the water permeability of her red cells was reduced to that of a lipid bilayer, whereas that of her husband was normal, and her children half normal (25).

We also showed that the protein was permeable only to water; urea, glycerol, ammonia, and, most notably protons, did not permeate (22, 23). The activation energy was 3 kCal/mole/°K, and the water flux was exquisitely sensitive to mercurials. When Thomas Walz and Peter Engel developed aquaporin crystal structures embedded in large liposomes, we demonstrated that these structures were entirely functional, with conductances identical to those of our originally reconstituted protein, and low activation energy (24). The finding that these crystal structures were functional opened the way for determination of the crystal structure of aquaporin 1, at high resolution.

In combination with crystal structures, molecular dynamics simulations have developed a detailed model of water permeation across the aquaporin pore (26, 27). This model depended heavily on the conductance data and the selectivity of the pore for water over other substances, especially protons. We helped validate the molecular dynamics approach by modeling the flux of deuterium oxide across the pore, predicting its permeability, and showing that the model accurately predicted the actual permeability (28). The structures of numerous aquaporins have now been, and molecular dynamics simulations have helped develop a clear understanding of how water and other substances cross them.

We also showed that aquaporin 1 can conduct gases such as CO_2 (29). This has led to a controversy in the literature regarding whether CO_2 can cross the pore, and, given the high permeability of gases across the membrane, whether such a flux is physiologically relevant (30–32). The controversy is fueled by the technical difficulty in making the measurements, and the high permeability of gases through lipid bilayers. Molecular dynamics simulations now predict that gases such as CO_2 become concentrated in, and can rapidly permeate, the highly hydrophobic central "doughnut hole" that lies between the four monomers in the aquaporin tetramer (reviewed in 27). Thus, it is likely that gases such as CO_2 do cross aquaporins. Recent studies by Garvin et al have indicated that NO can cross aquaporin 1; this may play a role in regulation of endothelial function (33, 34). In the case of CO_2, we and others have been unable to show an increase in gas flux when aquaporin 1 is included in lipid bilayers in Ussing chambers.

The combined methods of membrane protein reconstitution in lipid bilayers, careful biophysical studies of protein function, structural definition of protein structure, and molecular dynamics simulations have permitted a clear definition of how many aquaporins function at

an atomic level of resolution. These studies have provided a model for how to approach the definition of function of other membrane transporters. From such a detailed understanding it will be possible in the future to design specific inhibitors of transporter function, which may lead to important new approaches to the treatment of human diseases.

Studies in the Function of Other Aquaporins

Using yeast constructs, we functionally reconstituted aquaporin 2, which, in response to antidiuretic hormone (ADH), markedly increases water permeability of an otherwise impermeable collecting duct cell apical membrane, permitting the kidney to reabsorb free water so that the organ can maintain osmotic homeostasis (35). Early studies showed that aquaporin 2 is phosphorylated as a result of ADH action, and that ablation of the relevant serine by site-directed mutagenesis prevents the protein from trafficking to the apical membrane and from increasing water flux. To determine whether phosphorylation/dephosphorylation opens and closes the water pore, we maximally phosphorylated and dephosphorylated aquaporin 2 in membrane vesicles from a rat collecting duct and measured water permeabilities in each setting. Water flux was extremely rapid in these vesicles, but was entirely unchanged by phosphorylation state. These results indicate that, unlike the situation with many plant aquaporins, aquaporin 2 is not gated, meaning that the water channel is always open whether or not it is phosphorylated by ADH signaling, but instead it regulates water flow by trafficking into and out of the apical membrane (36).

Aquaporins in a Wide Array of Species

We have helped to define the roles of aquaporins in a number of organisms and tissues. In *archaebacteria,* the evolutionarily most ancient aquaporin, likely serves as a transporter of hydrogen sulfide (37). In soybean root nodules we defined the transport properties of Nodulin 26, which transports four carbon sugars into the symbiosome, fueling the fixation of nitrogen by rhizobacteriods that originate from the soil and are endocytosed to form the organelle (38, 39). We have identified and characterized several aquaporins in insects (40, 41). These help mosquito Malphigian tubules to excrete a salt and water ultrafiltrate of the blood that the animal is feeding on during feeding, so that the animal is light enough to fly away from the organism it has bitten. We have defined the functions of several fish aquaporins, including those of the shark rectal gland; these permit the organ to excrete a high salt ultrafiltrate of the plasma without allowing leakage of urea from the

animal (42, 43). Finally, we have helped characterize 36 aquaporins in the European Poplar, helping to determine how the tree transports water from its roots to the leaves (44).

Aquaporins in Human Disease

Humans express 13 aquaporins, including representatives of the "pure" aquaporins, which conduct water but not solutes, and the aquaglyceroporins, which transport solutes such as glycerol. Table 2 lists many, but not all of these aquaporins. As can be seen from the table, aquaporins may be involved in a number of human disease conditions, including cataracts (45, 46), diabetes insipidus/hyponatremia (47, 48), Sjogren's syndrome (49), brain edema after brain injury (50), and obesity (51), as regulated by glycerol metabolism between adipocytes and hepatocytes. Aquaporins are also involved in cell motility and wound healing (52). The ability to upregulate or inhibit aquaporin function selectively in specific tissues may lead to novel therapeutic approaches to human disease. To date, specific inhibitors suitable for clinical use have not been identified using high throughput screening approaches (52); however, our current knowledge of aquaporin pore structure/function relationships and the structure of the carboxy terminus of the molecule, which

TABLE 2
Relationship between Some Human Aquaporins and Diseases

Aquaporin	Location	What it Transports	Disease
AQP 0	Lens	Water, solutes	Cataracts
AQP 1	RBC Renal Tubule Endothelia	Water, NO	Angiogen. Mild DI
AQP 2	Collecting duct	Water	Severe DI
AQP 3	Collecting duct, gastric epithelial, skin, urothelial	Water, solutes	Severe DI Wound healing
AQP 4	Collecting duct airway, CNS secr. epithelia	Water	Brain edema
AQP 5	Lacrimal and salivary glands, alveoli	Water	Sjogren's syndrome
AQP 7	Adipocytes	Water, glycerol	Obesity?
AQP 9	Liver	Water, glycerol	Obesity?

Abbreviations: NO, nitrous oxide; DI, diabetes insipidus.

may play a regulatory role, should permit the development of "designer drugs" that can target specific aquaporins.

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DISCUSSION

May, Gainesville: Another name for apoptosis programmed cell death is shrinkage necrosis. It was called that before they got these other names. The cells shrink as they undergo intracellular destruction of themselves. Is the water channel involved at all in that shrinkage process, or is it just the loss of structural components inside the cell sort of collapsing it?

Zeidel, Boston: Well, likely it may well be that you've got leakage out of ions followed by leakage of water. A huge proportion of cells have water channels, so if the ions leak out and then the water follows, then that would account for the shrinking, and that can occur either through the waters channels or, in some cases, through the lipid bilayer of the membrane depending on its composition.

May, Gainesville: So, is there any evidence that affecting the function through some of these drugs, mercury or whatever, of the water channel could change whether a cell rapidly or slowly dies?

Zeidel, Boston: That is, at present, unknown. The only thing we do know is that there have been some studies looking at how cells migrate and it turns out that they put aquaporins up at the leading edge of the migration and reduction of that function can have an impact on the ability of cell to migrate and wound healing, but I don't think that anyone's really shown an effect on apoptosis.